REMARKS

Amendments

Claims 17 and 26-28 have been amended, and claims 1-16, 20-23, 25 and 31 have been canceled. Upon entry of the amendment, claims 17-19, 24 and 26-30 will be pending.

The specification has been amended to update cited application information. Previously cited U.S. Patent Application Ser. No. 08/971,310, filed November 17, 1997 (and now abandoned) was converted to 60/684,194, on which issued US patent no. 6,815,185 depends as a priority application filing. Contrary to the Examiner's assertions, the amendment does not recite new matter as the only document incorporated by reference is the disclosure of the originally cited 08/971,310 application. US patent no. 6,815,185 is only being cited as a publicly available document which contains the disclosure of the '310 application.

Rejections

Rejections under 35 U.S.C. § 101

Claims 17-19, 24 and 26-30 are rejected because the claimed subject matter allegedly is not supported by either a specific or substantial asserted utility or a well-established utility. Applicant respectfully traverses the rejection.

Amended claim 1 is drawn to transgenic mouse whose genome comprises a null allele of the endogenous low density lipoprotein-related protein 5 (LRP5) gene.

1. The Utility Requirement

Section 101 of the Patent Act of 1952, 35 U.S.C. § 101, provides that "whoever invents or discovers any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof," may obtain a patent on the invention or discovery.

According to the Federal Circuit:

> The threshold of utility is not high: An invention is "useful" under section 101 if it is capable of providing some identifiable benefit. See Brenner v. Manson, 383 U.S. 519, 534, 16 L. Ed. 2d 69, 86 S. Ct. 1033 (1966); Brooktree Corp. v. Advanced Micro Devices, Inc., 977 F.2d 1555, 1571 (Fed. Cir. 1992) ("To violate § 101 the claimed device must be totally incapable of achieving a useful result"); Fuller v. Berger, 120 F. 274, 275 (7th Cir. 1903) (test for utility is whether invention "is incapable of serving any beneficial end").

(*Juicy Whip v Orange Bang*, 185 F.3d 1364; 51 U.S.P.Q.2d 1700 (Fed. Cir. 1999)(emphasis added)).

2. Well-Established Utility

According to 35 U.S.C. § 101, "[w]hoever invents . . . any new and useful . . . composition of matter may obtain a patent therefore. . . . "

Under the Patent Office's Utility Requirement Guidelines:

> If at any time during the examination, it becomes readily apparent that the claimed invention has a well-established utility, do not impose a rejection based on lack of utility. An invention has a well-established utility if (i) a person of ordinary skill in the art would immediately appreciate why the invention is useful based on the characteristics of the invention (e.g., properties or applications of a product or process), and (ii) the utility is specific, substantial, and credible.

Applicant submits that in light of arguments of record, a person of ordinary skill in the art would immediately appreciate why the invention is useful. Thus, it cannot be reasonably debated that a person of ordinary skill in the art would not immediately appreciate why the invention is useful: for determining gene function.

3. Substantial Utility

The Examiner argues that the asserted utilities are not substantial.

According to the MPEP:

> A "substantial utility" defines a "real world" use. Utilities that require or constitute carrying out further research to identify or reasonably confirm a "real world" context of use are not substantial utilities. . . . the following are examples of situations that require or constitute carrying out further research to identify or reasonably confirm a "real world" context of use and, therefore, do not define "substantial utilities":
>
> (A) Basic research such as studying the properties of the claimed product itself or the mechanisms in which the material is involved;
>
> Office personnel must be careful not to interpret the phrase "immediate benefit to the public" or similar formulations in other cases to mean that products or services based on the claimed invention must be "currently available" to the public in order to satisfy the utility requirement. See, e.g., Brenner v. Manson, 383 U.S. 519, 534-35, 148 USPQ 689, 695 (1966). Rather, any reasonable use that an applicant has identified for the invention that can be viewed as providing a public benefit should be accepted as sufficient, at least with regard to defining a "substantial" utility.

(MPEP § 2107.01 I)(emphasis added).

The MPEP additionally provides:

> Office personnel must distinguish between inventions that have a specifically identified substantial utility and inventions whose asserted utility requires further research to identify or reasonably confirm. Labels such as "research tool," "intermediate" or "for research purposes" are not helpful in determining if an applicant has identified a specific and substantial utility for the invention.

(MPEP § 2107.01, I).

A use is not substantial where further research is required to identify any use. This is not the case in the present application. Knockout mice have a well-known use in the study of gene function. In the present case, the instant invention does not require further research to establish a utility. Applicant has determined that the LRP5 gene is associated with, for example, retinal degeneration, increased anxiety and hypoactivity. No further research is required to establish any use. The invention has a "real world use" – as demonstrated by the delivery of the claimed invention to at least one large pharmaceutical company (If the Examiner requires evidence of such sales and purpose of such use, Applicant shall so provide such evidence.) Whether additional research is required to identify therapeutic agents targeting the LRP5 gene or to further characterize the function of the LRP5 gene is irrelevant to whether the claimed invention has satisfied the utility requirement (see, for example, *In re Brana*, "Usefulness in patent laws . . . necessarily includes the expectation of further research and development.")

With regard to substantiality, according to the MPEP:

> **any reasonable use** that an applicant has identified for the invention that can be viewed as providing a public benefit **should be accepted as sufficient,** at least with regard to defining a "substantial" utility.

Certainly providing an *in vivo* model for studying the function of the LRP5 gene is a reasonable use.

In addition, the MPEP specifically cautions Examiners not to get confused by labeling inventions as research tools:

> Office personnel must distinguish between inventions that have a specifically identified substantial utility and inventions whose asserted utility requires further research to identify or reasonably confirm. Labels

> such as "research tool," "intermediate" or "for research purposes" are not helpful in determining if an applicant has identified a specific and substantial utility for the invention.

Applicant respectfully submits that the Examiner has done what the MPEP specifically cautions against, by providing: "[a]n assessment that focuses on whether an invention is useful only in a research setting thus does not address whether the invention is in fact "useful" in a patent sense."

4. Specific Utility

The Examiner states that the asserted uses are not specific.

According to the MPEP, "specific utility" means "specific" to the subject matter claimed as compared to a "general utility" that would be applicable to the broad class of the invention (MPEP 2107.01). Use of the LRP5 -/- and +/- mice to study the function of the LRP5 gene and the association of the LRP5 gene with, for example, retinal degeneration, increased anxiety and hypoactivity, is specific to this mouse. Even if there were many other genes associated with these phenotypes, only the LRP5 knockout mouse (as opposed to all other knockout mice) would be used to study the specific role of this gene in, for example, heart disease. The Examiner is respectfully requested to explain (1) how the asserted utility of determining the function of the LRP5 gene would be applicable to all other knockout mice; and (2) how the asserted use of studying the association of the LRP5 gene with retinal degeneration, increased anxiety and hypoactivity would be applicable to all other knockout mice. The Examiner is requested to explain how all other knockout mice would be used to study the function of the LRP5 gene.

5. In re Brana

Applicant submits that the legal principles as well as the facts of *Brana* are applicable to the present case. In *Brana*, the Board held that the applicant's specification failed to disclose a specific disease against which the claimed compounds were useful. The Federal Circuit reversed and held that the mouse tumor model represented a specific disease against which the compounds were effective. It is Applicant's position that a mouse demonstrating, for example, retinal degeneration, is sufficient to establish the animal's use as a model for eye diseases and disorders. As in *Brana*, confirmation of the phenotype in humans is unnecessary. In *Brana*, the

PTO was aware of the asserted use against the mouse tumor lines but did not find the use specific – as in the present case:

> Applicants' specification, however, also states that the claimed compounds have "a better action and a better action spectrum as antitumor substances" than known compounds, specifically those analyzed in Paull. As previously noted, see supra note 4, Paull grouped various benzo [de]isoquinoline-1,3-diones, which had previously been tested in vivo for antitumor activity against two lymphocytic leukemia tumor models (P388 and L1210), into various structural classifications and analyzed the test results of the groups (i.e. what percent of the compounds in the particular group showed success against the tumor models). Since one of the tested compounds, NSC 308847, was found to be highly effective against these two lymphocytic leukemia tumor models, 14 applicants' favorable comparison implicitly asserts that their claimed compounds are highly effective (i.e. useful) against lymphocytic leukemia. An alleged use against this particular type of cancer is much more specific than the vaguely intimated uses rejected by the courts in Kirk and Kawai. See, e.g., Cross v. Iizuka, 753 F.2d at 1048, 224 USPQ at 745 (finding the disclosed practical utility for the claimed compounds -- the inhibition of thromboxane synthetase in human or bovine platelet microsomes -- sufficiently specific to satisfy the threshold requirement in Kirk and Kawai.)
>
> The Commissioner contends, however, that P388 and L1210 are not diseases since the only way an animal can get sick from P388 is by a direct injection of the cell line. The Commissioner therefore concludes that applicants' reference to Paull in their specification does not provide a specific disease against which the claimed compounds can be used. We disagree.

(*Brana* at 1440). The court went on:

> The ultimate issue is whether the Board correctly applied the Section 112 Para.1 enablement mandate and its implicit requirement of practical utility, or perhaps more accurately the underlying requirement of Section 101, to the facts of this case. As we have explained, the issue breaks down into two subsidiary issues: (1) whether a person of ordinary skill in the art would conclude that the applicants had sufficiently described particular diseases addressed by the invention, and (2) whether the Patent Act supports a requirement that makes human testing a prerequisite to patentability under the circumstances of this case.
>
> The first subsidiary issue, whether the application adequately described particular diseases, calls for a judgment about what the various representations and discussions contained in the patent application's specification would say to a person of ordinary skill in the art. We have considered that question carefully, and, for the reasons we explained above in some detail, we conclude that the Board's judgment on this question was erroneous. Our conclusion rests on our understanding of what a person skilled in the art would gather from the various art cited, and from the statements in the application itself. We consider the Board's error to be sufficiently clear that it is reversible whether viewed as clear error or as resulting in an arbitrary and capricious decision.

> The second subsidiary issue, whether human testing is a prerequisite to patentability, is a pure question of law: what does the practical utility requirement mean in a case of this kind. Under either our traditional standard or under the APA standard no deference is owed the Agency on a question of law, and none was accorded.
>
> If the question concerning the standard of review, raised by the Commissioner, is to be addressed meaningfully, it must arise in a case in which the decision will turn on that question, and, recognizing this, the parties fully brief the issue. This is not that case. We conclude that it is not necessary to the disposition of this case to address the question raised by the Commissioner; accordingly, we decline the invitation to do so.

(*Brana* at 1443-44). The court's position is reflected in the MPEP: if an "assertion would be considered credible by a person of ordinary skill in the art, do not impose a rejection based on lack of utility" (MPEP § 2107, II (A)(3); II (B)(1)). If it is well known to those skilled in the art that knockout mice are useful for studying gene function, then those skilled in the art would certainly regard such use as credible, specific and substantial. Nothing more is required to satisfy the statutory requirement. Applicant submits that, as in *Brana*, one skilled in the art would find the asserted use credible, substantial and specific.

In *Brana*, the court found a mouse with an implanted murine tumor to represent a specific disease. Applicant submits that the claimed mouse having with a null allele of the endogenous LRP5 gene and demonstrating retinal degeneration, increased anxiety and hypoactivity, likewise represents a specific disease.

6. Additional Examiner Arguments

The Examiner argues at length that the observed phenotypes are not specific to any disease.

The issue is not whether the test is specific for a disease but whether the asserted use is specific for the claimed invention.

The Examiner argues that the function of the gene may never be determined from the knockout mouse (p. 5).

Applicant is not claiming a gene. Applicant is claiming a mouse having a null LRP5 gene.

With regard to Austin, the NIH citation, the Examiner argues that the references were published well after the present application was filed.

The Examiner cites no legal support why Austin and NIH references should not be considered. The references are not being cited to support a post-filing assertion of utility. The goal of determining gene function is clearly set forth in the specification. Austin supports this statement. Moreover, courts have accepted post-filing activities to support an asserted utility. For example, in *In re Brana*, the application's filing date was June 30, 1988. The applicants relied on an affidavit submitted June 19, 1991 to provide evidence of the compounds activity, *a date well after the filing date*. The Federal Circuit noted:

> Enablement, or utility, is determined as of the application filing date [citations omitted]. The Kluge declaration, though dated after applicants filing date, can be used to substantiate any doubts as the asserted utility since this pertains to the accuracy of a statement already in the specification. [citations omitted] It does not render an insufficient disclosure enabling, but instead goes to prove that the disclosure was in fact enabling when filed (i.e., demonstrated utility).

(*Brana*, n.19). Thus, Austin and the NIH citation should have been considered by the Examiner as evidence supporting the utility of the claimed invention.

As noted by the Examiner, Austin states knockout mice "can" be used to elucidate gene function. Therefore, the asserted use is credible.

The Examiner cites *Schoenwald* for the proposition that providing evidence that a product was known in the art was not evidence that the product had patentable utility (p. 12).

Schoenwald does not stand for the proposition cited by the Examiner. In fact, the utility requirement was not at issue. The case involved the novelty of a claimed composition that was described in a prior art reference. The court held that the reference need not recite a utility in order to anticipate the claimed composition.

The Examiner argues that the phenotype of the mouse is "not necessarily" reflective of the knocked out gene.

Applicant had responded previously to this Examiner argument by citing Doetschman, to which the Examiner has failed to respond:

"In addition, as pointed out by Doetschman, one clearly skilled in the art, (*Laboratory Animal Science* 49:137-143, 137 (1999)(copy attached), the phenotypes observed in mice do correlate to gene function:

> The conclusions will be that the knockout phenotypes do, in fact, provide accurate information concerning gene function, that we should let the unexpected phenotypes lead us to the specific cell, tissue, organ culture, and whole animal experiments that are

relevant to the function of the genes in question, and that the absence of phenotype indicates that we have not discovered where or how to look for a phenotype.

(emphasis added)."

The Examiner argues that the specification does not teach that the transgenic mice were compared with mice of the same background, and that the ES cell could be the cause of the phenotype.

Applicant does not agree. According to the specification:

> The homozygous mice demonstrated eye abnormalities, including retinal regeneration.
>
> Specifically, histopathology studies demonstrated that the eyes of the homozygous mice suffered from retinal degeneration, including bilateral retinal regeneration. In each homozygous mutant, at least one of the following retinal changes were present: retinal folds; thinning and vacuolation of the pigment epithelium layer; degeneration of photoreceptors; thinning, disorganization, and pyknosis of the outer nuclear layer; thinning and disorganization of the outer plexiform layer, including juxtaposition of the photoreceptor nuclei and the bipolar cell or inner nuclear layer; disorganization of the inner nuclear layer; thinning of the inner plexiform layer; loss of ganglion cell nuclei, especially large ganglion cells; and, gliosis of the nerve fiber layer. The changes were generally more prominent in the outer layers of the retina (photoreceptor layers) and least pronounced in the inner layers (inner nuclear layer, inner plexiform layer, ganglion cell layer, and nerve fiber layer).

(Example 1).

According to the Examiner's logic, 129/OlaHsd wild-type mice would exhibit retinal degeneration. The Examiner is requested to provide support, in the form of scientific publication, that 129 mice spontaneously exhibit retinal degeneration.

With regard to the other observed phenotypes, one skilled in the art would understand that the mice were compared with mice of same background. According to the specification:

> A statistical analysis of the various behaviors measured can be carried out using any conventional statistical program routinely used by those skilled in the art (such as, for example, "Analysis of Variance" or ANOVA). A "p" value of about 0.05 or less is generally considered to be statistically significant, although slightly higher p values may still be indicative of statistically significant differences. To statistically analyze abnormal behavior, a comparison is made between the behavior of a transgenic animal (or a group thereof) to the behavior of a wild-type mouse (or a group thereof), typically under certain prescribed conditions. "Abnormal behavior" as used herein refers to behavior exhibited by an animal having a disruption in the Lrp5 gene, *e.g.* transgenic animal, which differs

> from an animal without a disruption in the Lrp5 gene, *e.g.* wild-type mouse. Abnormal behavior consists of any number of standard behaviors that can be objectively measured (or observed) and compared. In the case of comparison, it is preferred that the change be statistically significant to confirm that there is indeed a meaningful behavioral difference between the knockout animal and the wild-type control animal. Examples of behaviors that may be measured or observed include, but are not limited to, ataxia, rapid limb movement, eye movement, breathing, motor activity, cognition, emotional behaviors, social behaviors, hyperactivity, hypersensitivity, anxiety, impaired learning, abnormal reward behavior, and abnormal social interaction, such as aggression.

(page 19)(emphasis added). The term "wild-type control mouse" is a standard term used in the art which refers to a strain, age, and gender matched (+/+) mouse. As explained by Crawley (*What's Wrong with My Mouse? Behavioral Phenotyping of Transgenic and Knockout Mice*, Wiley-Liss 2000) (copy of relevant page attached):

> Identified F_1 heterozygote offspring are mated with each other to produce an F_2 generation. Theoretically, the F_2 population with follow the principles of Mendelian segregation, resulting in one-fourth (1/4) homozygous mutants (-/-), one-half (2/4) heterozygotes (+/-), and one-fourth (1/4) homozygous wildtype controls (+/+).

(p. 15)(emphasis added).

Thus, one skilled in the art would understand that wild-type controls were strain matched.

With regard to background, the Examiner argues that the mixed strain knockout mouse was not adequately backcrossed to be compared to a C57BL/6.

The knockout mice and the "wild-type" control mice were of identical background. As described in Example 1:

> F1 mice were generated by breeding with C57BL/6 females. The resultant F1N0 heterozygotes were intercrossed to produce F2N0 homozygotes, or were backcrossed to C57BL/6 mice to generate F1N1 heterozygotes.

(Example 1). Thus, the resulting homozygous F2N1 knockout mice and the wild-type controls were 25% 129 and 75% BL/6. As argued previously (see previous response, page 15), and apparently overlooked by the Examiner, Figure 5 clearly shows F2N1 (-/-) males compared with F2N1 (+/+) controls.

With regard Jiao, it is irrelevant whether Jiao was available at the time of filing. The Examiner's argument that one must have an established association with regard to the function of

the gene in a human prior to creating a knockout in order to satisfy the utility requirement is simply unfounded. As argued previously argued, Jiao supports the utility of the claimed invention as evidence of one skilled in the art actually using the claimed invention.

With regard to Olsen, Bowery and Mombereau, Applicant cites arguments made previously.

The Examiner argues "patentable utility requires that one of skill successfully use the product for its asserted use."

Applicant requests the Examiner to define "successfully use" and to provide legal support for this apparent additional requirement. Applicant has created the mouse and has actually used the mouse in numerous physiological and behavioral tests. The mouse has been actually provided to at least one large pharmaceutical company. Applicant has determined that the mouse has certain eye abnormalities which associates the gene with retinal disorders. This observation has been duplicated by Jiao, which the Examiner conveniently dismisses as post-filing.

6. Summary

In summary, Applicant submits that the claimed transgenic mouse, regardless of any disclosed phenotypes, has inherent and well-established utility in the study of the function of the gene, and thus satisfies the utility requirement of section 101. Moreover, Applicant believes that the transgenic mice are useful for studying LRP5 gene function with respect to the cited phenotypes, for studying gene expression, and are therefore useful for a specific practical purpose that would be readily understood by and considered credible by one of ordinary skill in the art.

In light of the arguments set forth above, Applicant does not believe that the Examiner has properly made a *prima facie* showing that establishes that it is more likely than not that a person of ordinary skill in the art would not consider that any utility asserted by the Applicant to be specific and substantial. (*In re Brana*; MPEP § 2107).

According to the Federal Circuit: "The threshold of utility is not high" (cited above). Applicant submits that the Examiner has established his own threshold of patentable utility supported neither in the Utility Guidelines nor under existing federal case law.

Rejection under 35 U.S.C. § 112, first paragraph

Claims 17-19, 24 and 27-30 are rejected because one skilled in the art would allegedly not know how to use the claimed invention as a result of the alleged lack of either a specific or substantial asserted utility or a well-established utility for the reasons set forth in the utility rejection. Applicants respectfully traverse the rejection. For the reasons set forth above, the claimed invention satisfies the utility requirement. Therefore, one skilled in the art would know how to use the invention.

The Examiner argues that the specification does not provide a nexus between the disruption and the observed phenotypes.

Applicants created a knockout mouse with a single gene disrupted. Applicant compared this mouse to a control mouse of the same strain. The knockout mouse demonstrated phenotypes not observed in the control mouse. Applicant has established a link between the phenotypes and the gene. Applicant once again reminds the Examiner that Applicant is claiming a mouse, not a gene.

Rejection under 35 U.S.C. § 112, first paragraph

Claims 17-19, 24 and 26-30 are rejected for allegedly failing to comply with the written description requirement.

The Examiner argues that the phrase "null LRP5 allele" is new matter.

Applicant disagrees. According to the Federal Circuit:

> The test for determining compliance with the written description requirement is whether the disclosure of the application as originally filed reasonably conveys to the artisan that the inventor had possession at that time of the later claimed subject matter, rather than the presence or absence of literal support in the specification for the claim language.

In re Kaslow, 707 F.2d 1366, 217 USPQ 1089 (Fed.Cir.1983).

The Applicants were clearly in possession of a mouse with a null LRP5 allele. According to Hasty (*Gene Targeting, Principles, and Practice in Mammalian Cells* in Joyner, Gene Targeting: *A Practical Approach*, Oxford University Press 2000) (copy of relevant pages previously provided):

> Since the most common experimental strategy is to ablate the function of a target gene (null allele) by introducing a selectable marker gene . . .
>
> Considering that products from the mutated locus may have some function (normal or abnormal) it is important to design a replacement vector so that the targeted allele is null,

particularly in the absence of a good assay for the gene product. Disruption or deletion of the coding sequence by the positive selection marker will in most instances ablate a gene's function.

(pp. 1, 5)(emphasis added). Example 1 provides:

> To investigate the role of LRP5, disruptions in genes comprising the sequence set forth in SEQ ID NO:1 were produced by homologous recombination. More particularly, as shown in Figure 3A-3C, a specific targeting construct having the ability to disrupt or modify genes, specifically comprising SEQ ID NO:1 was created using as the targeting arms (homologous sequences) in the construct, the sequences identified herein as SEQ ID NO:3 and SEQ ID NO:4.

Figures 3A, B and C clearly show insertion of a *Neo* gene into the coding sequence of the LRP5 gene. According to Hasty, such a disruption would be expected to result in a null allele.

The term "exogenous DNA" has been deleted.

The term "selection marker" has been amended to recite "positive selection marker."

The term "lacO sites" is recited on page 10, lines 14-19.

Withdrawal of the rejections is respectfully requested.

Rejections under 35 U.S.C. § 112, 2nd paragraph

Claims 17-19, 24 and 26-30 stand rejected as allegedly indefinite.

The Examiner asserts that " LRP5 gene" is indefinite.

As amended, the claims recite a transgenic mouse whose genome comprises a null allele of the endogenous LRP5 gene. One skilled in the art would clearly understand that the recited gene is the mouse LRP5. The broader definition cited by the Examiner is a non-species specific definition applicable to any transgenic animal.

The Examiner asserts the term "null LRP5 allele" is indefinite.

Applicant disagrees. As amended, the claims encompass a transgenic mouse whose genome comprises a null allele of the endogenous LRP5 gene. The specification provides that "[t]ransgenic animal" refers to an animal that contains within its genome a specific gene that has been disrupted or otherwise modified or mutated by the method of gene targeting. "Transgenic animal" includes both the heterozygous animal (*i.e.*, one defective allele and one wild-type allele) and the homozygous animal (*i.e.*, two defective alleles)(cited above). It is clear from the claim that one or both alleles have been disrupted.

The term "null" is well understood in the art as meaning ablating the function of that allele. For example,

According to Genes VII (Lewin, Oxford University Press (2000)) (copy of relevant pages attached):

> The converse of the introduction of new genes is the ability to disrupt specific endogenous genes. Additional DNA can be introduced within a gene to prevent its expression and to generate a null allele. Breeding from an animal with a null allele can generate a homozygous "knockout", which has no active copy of the gene. This is a powerful method to investigate directly the importance and function of the gene.

(p. 508)(emphasis added).

According to Hasty (*Gene Targeting, Principles, and Practice in Mammalian Cells* in Joyner, Gene Targeting: *A Practical Approach*, Oxford University Press 2000) (copy of relevant pages previously provided):

> Since the most common experimental strategy is to ablate the function of a target gene (null allele) by introducing a selectable marker gene . . .
>
> Considering that products from the mutated locus may have some function (normal or abnormal) it is important to design a replacement vector so that the targeted allele is null, particularly in the absence of a good assay for the gene product. Disruption or deletion of the coding sequence by the positive selection marker will in most instances ablate a gene's function.

(pp. 1, 5)(emphasis added).

According to Crawley (What's Wrong With My Mouse *Behavioral Phenotyping of Transgenic and Knockout Mice*, Wiley-Liss 2000) (copy of relevant pages attached):

> **Knockout mice** have a **gene deleted**. The **null mutant homozygous** knockout mouse is deficient in both alleles of a gene, the **heterozygote** is deficient in one of its two alleles for the gene. The **genotype** is -/- for the null mutant, +/- for the heterozygote, and +/+ for the wildtype normal control.

(p. 2)(emphasis in original).

As the term "null allele" is clearly recognized in the art, the claim satisfies the definiteness requirement.

Rejection under 35 U.S.C. § 102(b)

Claims 24, 26-28 and 30 stand rejected as being anticipated by Rohlmann (1996 and 1998). Rohlmann discloses a conditional KO mouse having a liver specific disrupted LRP allele.

The claims as amended are drawn to a transgenic mouse whose genome comprises a null allele of the endogenous low density lipoprotein-related protein 5 (LRP5) gene. Rohlmann does not disclose a mouse whose genome comprises a null LRP5 allele. A clear indication that LRP is not LRP5 is the fact that an attempt to create a mouse having a null LRP gene resulted in an embryonic lethal (Rohlmann, p. 1562; p. 689). The references do not anticipate the claimed invention. Withdrawal is requested.

Rejection under 35 U.S.C. § 103(a)

Claims 24, 26-28 and 30 stand rejected as obvious over Rohlmann in view of Hey. Hey is cited as teaching the amino acid sequence of mouse LRP5 protein.

Applicant respectfully traverses the rejection.

To establish a *prima facie* case of obviousness, three basic criteria must be met. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations. Applicant respectfully submits that the Office Action fails to establish a prima facie case of obviousness because there is no reasonable expectation of success to that which Applicant has done by modifying the cited references. Moreover, Applicant respectfully submits that the combination of the references fails to teach or suggest all the claimed subject matter.

As a preliminary matter, Applicant questions how the Examiner can argue that the requisite motivation exists to create the claimed subject matter, when the Examiner argues above that the claimed subject matter has no patentable utility and that one skilled in the art would not know how to use the claimed subject matter. The Examiner is missing the point made by Applicant, and has not directly addressed the issue: why would one skilled in the art be motivated to make an invention if the invention was useless. The Examiner's position is clearly contradictory.

The claims, as amended, recite a transgenic mouse whose genome comprises a null allele of the endogenous LRP5 gene. Rohlmann teaches away from creating a mouse having a null

LRP5 gene, as the LRP gene disrupted mouse was embryonic lethal. In addition, the fact that the claimed mouse was viable would have been an unexpected result. Moreover, Rohlmann teaches how to make a site-specific conditional knockout, i.e, a non-genomic disruption. (Note that the conditional mouse is phenotypically normal, other than the liver specific site disruption). Applicant is claiming a mouse having a genomic disruption. Thus, even assuming motivation to combine, Rohlmann and Hey combined would not lead one to the claimed invention. Withdrawal is requested.

Claims 24 and 26-30 stand rejected as allegedly being obvious over Signorini in view of Hey. Signorini is cited as disclosing a mouse having a heterozygous and homozygous disruption in a GIRK2/Kir3.2 gene by inserting neo into the gene. The Examiner argues that it would have been obvious to disrupt a gene as taught by Signorini wherein the gene was the LRP5 gene.

Applicant submits that the cited references are not enabling or operative to produce the claimed transgenic mouse because neither reference teaches or suggests how to make the claimed invention. The GIRK2 deficient mouse disclosed by Signorini requires knowledge of the genomic sequence and restriction mapping of the GIRK2 gene (see Materials and Methods) in order to create a targeting vector. Hey discloses a protein sequence. Without a teaching of the genomic sequence or restriction mapping of the LRP5 gene, or how to create a null LRP5 allele, or how to create a targeting vector, the cited references do not enable one of ordinary skill in the art to do that which Applicant has done, and therefore would not have a reasonable expectation of success in making the claimed transgenic mouse.

Withdrawal is respectfully requested.

In view of the above amendments and remarks, Applicant respectfully requests reconsideration and a Notice of Allowance. If the Examiner believes a telephone conference would advance the prosecution of this application, the Examiner is invited to telephone the undersigned at the below-listed telephone number.

In the event a petition for an extension of time is required, this paper is to be considered such a petition. The Commissioner is hereby authorized to charge any deficiency or credit any overpayment to Deposit Account No. **502775**.

Respectfully submitted,

8-15-05
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Of Unicorns and Chimeras

Targeted gene mutation technologies began in the 1980s (Costantini and Lacy, 1981; Gordon and Ruddle, 1981; Harbers et al., 1981; Wagner et al., 1981a, 1981b; Jaenisch, 1988; Pascoe et al., 1992; Doetschman, 1991; Smithies, 1993; Bronson and Smithies, 1994; Smithies and Kim, 1994; Capecchi, 1989, 1994). The first big success in detecting a phenotype relevant to behavior in a transgenic mouse appeared in 1982. The cover illustration of the December 16th issue of *Nature*, shown here, excited the popular imagination with the dramatic results of the elegant experiments by Richard Palmiter and co-workers at the University of Washington (Palmiter et al., 1982). A growth hormone overexpressing transgenic mouse was much larger than normal littermate control mice of the same age and gender, showing more rapid weight gain. Successes multiplied in manipulations of the genomes of yeast and fruit flies. Technical advances allowing targeted gene mutation in mammals raised hopes that the new technology could be applied to discovering the role of individual genes in normal and abnormal behavioral processes. In the 1990s, this dream moved into the realm of reality. The present chapter provides an overview of the methods currently in common use for targeted gene overexpression in transgenic mice and targeted gene deletion in knockout mice. Original literature cited and review articles listed at the end of this chapter provide more in-depth information for the interested reader. The information at the end of this chapter includes background readings on DNA constructs, breeding strategies, mouse handbooks, companies offering breeding and genotyping services, and companies providing behavioral phenotyping services. Web site addresses relevant to these topics are provided.

TARGETING VECTOR

The process of developing a transgenic or knockout mouse begins with the cDNA for the gene. If the gene has not yet been cloned, or the full-length cDNA has not yet been accurately sequenced, a useful targeting vector cannot be designed.

Transgenic is defined by the **addition of a gene**. Transgenic mice may have a new gene added, for example, the human gene for a hereditary disease such as Huntington's (Carter et al., 1999), or an extra copy of an existing gene, for example, corticotropin releasing factor, to investigate excessive expression of a hormone (Stenzel-Poore et al., 1994). Transgenic techniques involve microinjection of the transgene within the DNA construct into the pronucleus of a fertilized mouse oocyte. The egg cell is large enough to be seen under the microscope. However, injecting the oocytes is a difficult and laborious process. An expert with very steady hands and excellent eye–hand coordination is able to transfer DNA successfully into a large number of oocytes. The success rate of the technique is proportional to the number of eggs injected since homologous recombinations are random, infrequent events. The more lottery tickets you buy, the better your chances of winning.

Transgenic methods are diagrammed in Figure 2.1. The process begins with the development of the fusion gene construct in which the cDNA transgene is driven by a carefully selected promoter sequence. Female mice are treated with hormones to induce superovulation. Large numbers of eggs are harvested. The fusion gene construct is microinjected directly into the nucleus of the oocyte, through a very fine needle, under a high-magnification microscope. The microinjected oocytes are then implanted into the oviducts of genetically

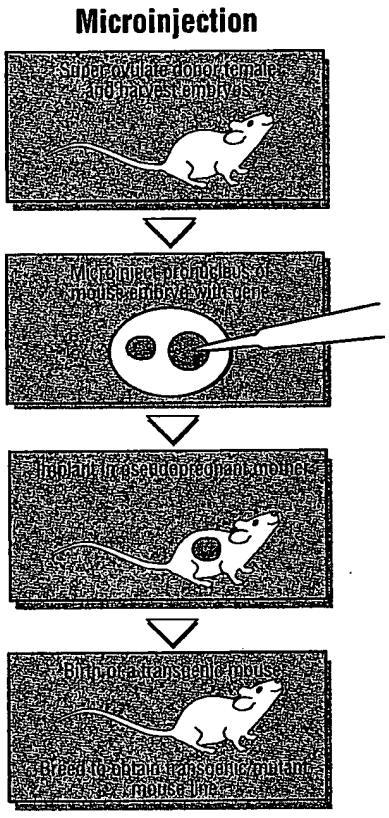

Figure 2.1 *Transgenic mice are generated by inserting a foreign gene, or an extra copy of a gene, into the pronucleus of a fertilized egg.* [From Taconic Farms, Inc., *Research Animal Review* (1998) 1(5): 2.]

normal, healthy female mice that have been pretreated with hormones to induce pseudopregnancy. Through random homologous recombination events, the fusion gene construct becomes integrated into the genome. When the transgene is integrated into the genome before the first cell division, the embryo develops with the foreign gene contained in every somatic cell and germ-line (sperm and egg) cell. When the transgene is integrated during subsequent cell divisions, the embryo develops as a genetic mosaic, with some cells containing the foreign gene and other cells normal. The mouse that develops from each microinjected egg is the **founder** of the mutant line.

The transgene is likely to integrate into a different chromosomal location in the genome in each founder mouse. As described in Chapter 12, current technology attempts to define the integration site, using tissue-specific promoters. With this conditional mutation technique, the site of incorporation of the transgene is determined by the specific promoter. The modified DNA construct inserts at a chromosomal location homologous with the cDNA of the promoter. For example, the CaMKIIα promoter limits expression of a targeted gene mutation to neurons of the forebrain (Mayford et al., 1996).

The transgene inserts into only one chromosome. The embryo thus develop as a heterozygote for the transgene. The heterozygotes are then mated with each other to generate homozygous transgenics, heterozygotes, and wildtype littermate controls, following a 1:2:1 Mendelian ratio. The DNA construct also contains a reporter gene, such as β-galactosidase (*lacZ*), with a nuclear localization signal (NLS). The reporter gene is simultaneously driven by the promoter for the transgene. *LacZ*-positive cells indicate the presence of the transgene in the cell. Concentrations of the reporter gene, the transgene, and the gene product are assayed to determine the overexpression level of the gene in the tissue of interest. Anatomical mapping of the localization of the reporter gene, the transgene, and the gene product is performed to describe the anatomical distribution of the expression of the transgene or to more precisely determine the neurons expressing a known gene product during stages of development (Itoh et al., 1998).

Targeted gene mutation is the technique designed to delete or inactivate a specific gene. **Knockout** mice are defined by a mutated gene which is no longer expressed. Knockout mice are generated by a different set of techniques than transgenic mice. Instead of a gene insertion, knockouts have a mutation introduced into a carefully chosen exon of the cDNA of the gene. The mutation is usually a selective deletion of a portion of DNA that is critical for the expression of the gene product. The deletion also usually shifts the reading frame for the DNA, rendering incorrect reading of the triplet base pair codes for the amino acids comprising the gene product.

The gene for resistance to an antibiotic drug is generally added to the DNA construct as a marker. A commonly used antibiotic resistance gene is the **neomycin resistance gene** (*Neo*^{r}). The cDNA for the *Neo*^{r} gene is inserted at a critical coding region of the targeted gene. Removal of a key sequence of DNA from the gene being "knocked out," insertion of the neomycin resistance tag, and the resulting disruption of the reading frame of the targeted gene, constitute the **targeted gene construct.** A typical **targeting vector** is shown in Figure 2.2.

The targeted gene construct is inserted into the genome of **embryonic stem cells.** Embryonic stem cells **(ES cells)** are lines of cultured cells, originally harvested from the inner cell mass of blastocysts. Because these cells are derived at a very early stage of development, they are pluripotent. All of their genes can be activated, all of their gene products can be synthesized, and the cells have the ability to differentiate into all types of tissues. The ES cell lines most frequently used for generating knockout mice are derived from the

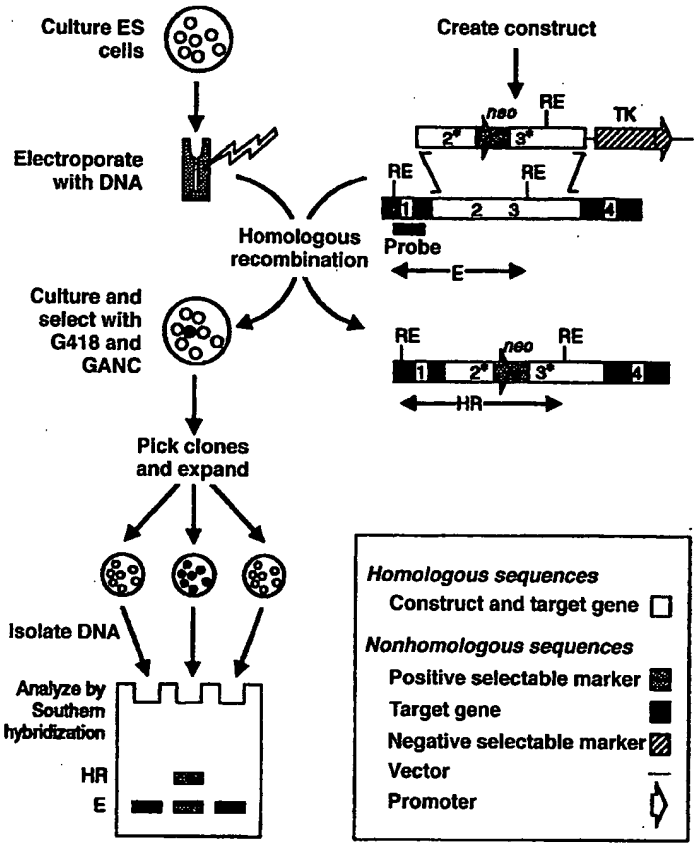

Figure 2.2 *Production, selection and identification of targeted gene disruption by homologous recombination. An example of a restriction enzyme (RE) and hybridization probe that can be used to identify cells in which homologous recombination has occurred (shaded colony) is shown. The predicted size of the restriction fragment generated from an unaltered target gene (E) and a target gene that has undergone homologous recombination (HR) is shown. If equal amounts of DNA are present in the lanes of the Southern blot, the intensity of each of the two hybridization fragments from the DNA of a homologous recombinant clone will be half of the intensity of the hybridizing fragment from unaltered clones.* [From Ausubel (1995), p. 9.16.2.]

129 inbred strains of mice. For unknown reasons, 129 substrains produce ES cells that are more likely to remain viable through the electroporation and implantation processes than ES cells from other mouse strains or from rats strains (Simpson et al., 1997).

Electroporation is the process in which the targeted gene construct is inserted into ES cells (Potter, 1995). The construct DNA is mixed with a suspension of ES cells in an apparatus that delivers electrical current to the electroporation plate. The current opens "pores" or channels in the ES cell membrane nucleus that are sufficiently large to permit entry of the DNA comprising of the targeted gene construct. When the current is turned off, the membrane pores close. The ES cell membrane is returned to its normal state, apparently undamaged. The DNA construct diffuses through the cytoplasm and enters the nucleus.

Random events of homologous recombination then occur within the ES cell nuclei. A very small percentage of ES cells, about one in a million, incorporate the targeted gene construct into the genome. Finding the ES cells that contain the mutation sounds like a needle-in-the-haystack search. The antibiotic resistance marker gene locates those rare ES cells containing the cDNA construct. All of the ES cells that underwent electroporation are grown in tissue culture containing a solution of the antibiotic, for example, neomycin, at concentrations sufficient to kill the cultured cells. The ES cells that do not contain the

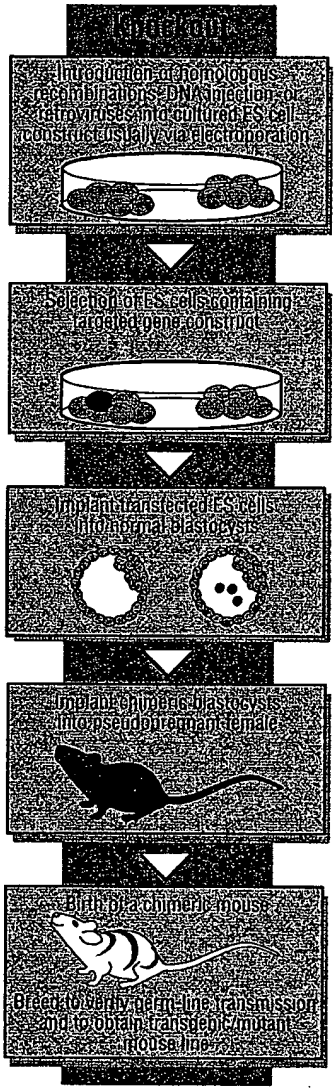

Figure 2.3 *Knockout mice are generated by inserting the mutated cDNA into embryonic stem cells, injecting the transfected cells into normal blastocysts and implanting the injected blastocysts into pseudopregnant normal female mice.* [From Taconic Farms, Inc. (1998) *Research Animal Review* 1(5): 2.]

targeting vector do not express neomycin resistance. The 999,999 failures are killed by the antibiotic. Only the ES cells that express the neomycin resistance gene product will successfully resist the antibiotic in the tissue culture medium, survive, and grow into colonies. Visible colonies of ES cells growing after the neomycin treatment are harvested as candidates likely to contain the mutated gene in their genome.

Positive ES cells are implanted into **blastulas** from normal mice. Blastulas are collected from superovulated female mice, often of the C57BL/6 inbred strain. Collection is usually performed at embryonic day 3, when the blastula is at a very early stage of development. A fine-gauge needle is used to microinject ES cells from the tissue culture colony into the central hollow blastocoele of each blastula. The injected blastulas are then implanted into **pseudopregnant female recipients**. These adoptive mothers can be of any strain. C57BL/6J and CD-1 are often used as recipients, as they sustain pregnancies well and provide good parental care. The stages of the knockout technology are diagrammed in Figure 2.3.

THE CHIMERA

Successful microinjection of ES cells into the blastocoele results in an embryo comprised of cells from the original blastula plus the ES cells implanted into the blastula. Cells of both origins grow in concert to form one complete embryo. The resulting mouse pup has tissues and organs composed of a mosaic mixture of cells derived from the original 129 ES cells and the original C57BL/6 blastula cells. The pup is called a **chimera** because it contains cells from two independent sources.* The C57BL/6 strain has black fur. The 129 strain has light grayish-brown fur. The coat color of chimeric pups is grayish brown, sometimes a mosaic of black and grayish-brown patches or stripes. If no ES cells were incorporated at the blastula stage, the pup will show a black coat color. The appearance of the fur is a useful early marker of a successful mutation. Chimeras are identified as soon as coat color is detectable, at about 3 weeks after birth.

F_1 AND F_2 OFFSPRING

The targeted gene construct is incorporated into the blastula at random locations. When incorporation is only in the somatic cells that develop into nonreproductive tissues, the original chimeras express the mutation, but their offspring do not. When incorporation is in cells of the blastula that develops into germ-line gametes, that is, eggs and sperm, the targeted mutation is transmitted to the next generation of the offspring of the chimera. The scheme for generating chimeras and their offspring is shown in Figure 2.4.

To detect germ-line transmission, a test cross is conducted. The chimera is bred to a mouse of a normal inbred strain, such as C57BL/6J. The F_1 offspring of the test cross are analyzed for expression of the mutation. An F_1 offspring that receives the mutated gene from the chimeric progenitor parent is heterozygous for the mutated gene. Southern blot analysis or polymerase chain reaction assay is performed on a small tissue sample from the

*In Greek mythology, the Chimera was a fire-breathing monster: a lion in the front, a goat in the middle, and a serpent in the back. Bellerophon killed the Chimera by thrusting lead down its throat. Its fiery breath melted the lead, which trickled down into the stomach and killed the Chimera, saving the Kingdom of Lycia (D'Aulaire and D'Aulaire, 1962).

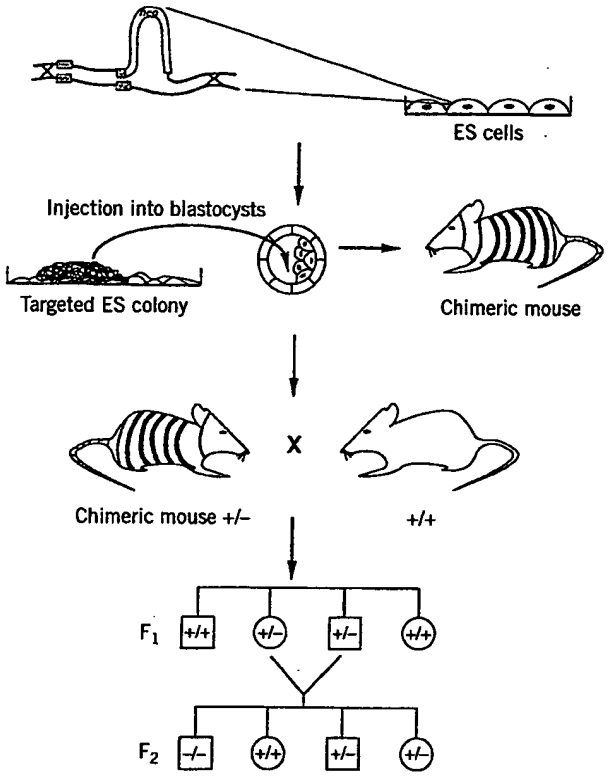

Figure 2.4 Chimeric mice grow from the blastulas containing embryonic stem cells expressing the targeted gene construct. When the mutation is expressed in germ-line cells of the chimera, the mutation is transmitted to subsequent generations. [From Wehner and Silva (1997), p. 244.]

tail of the offspring, to identify positive heterozygotes. Each positive heterozygote is a potential founder for a line of mutant mice.

Identified F_1 heterozygote offspring are mated with each other to produce an F_2 generation. Theoretically, the F_2 population will follow the principles of Mendelian segregation, resulting in one-fourth (1/4) homozygous mutants (−/−), one-half (2/4) heterozygotes (+/−), and one-fourth (1/4) homozygous wildtype controls (+/+). If the gene is lethal, the homozygotes will not survive. If the gene is located on the X or the Y chromosome, gender issues influence the ratio of males and females for each genotype.

The genotypes of the F_2 mice are tested by Southern blot for the presence of the normal gene in the +/+ mice, the presence of half the normal complement of the gene in the +/− mice, and the absence of the gene in the −/− mice. The expression of the gene product, when the gene product is known, is assayed by an appropriate technique, such as high-pressure liquid chromatography for an enzyme. The heterozygotes (+/−) may express half the gene product, reflecting the presence of half the gene dose, or may express variable amounts of the gene product in some cases. The homozygous mutant mice (−/−) should show no gene product. These confirmed −/− mice are termed the **null mutants**. Techniques for confirmation of the mutation are illustrated in Figure 2.5.

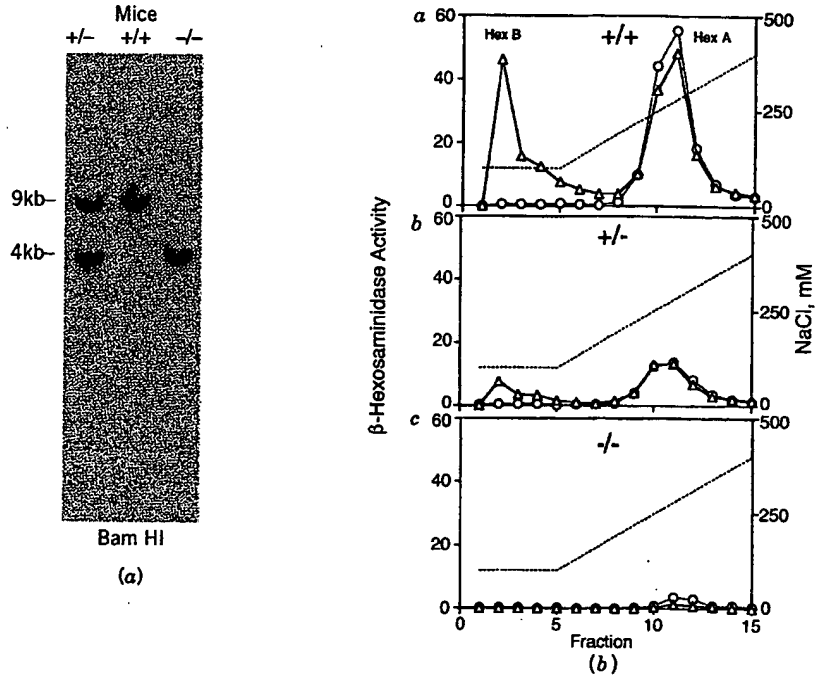

Figure 2.5 *Confirmation of the mutation in* Hexa *and* Hexb *knockout mouse models of Tay-Sachs and Sandhoff diseases. (a) Southern blot showing the absence of a band at 9 kb, from* Hexb *−/− mice deficient in the* HexB *gene. (b) High-pressure liquid chromatograph showing* Hexa *and* Hexb *gene products in wildtype controls, the absence of the* Hex A *peak in the fraction from the* Hexa *−/− knockouts, and the absence of the* Hex B *peak in the fraction from the* Hexb *−/− knockouts. Both peaks are absent in the double knockouts, representing the complete lack of β-hexosaminidase enzyme activity.* [From Sango et al. (1995), p. 171.]

Conventional gene disruption technology is now widely used in laboratories around the world. New modifications are becoming available. Tissue-specific **conditonal mutations** allow the investigator to insert the mutation only into specific cell types. **Inducible mutations** allow the investigator to turn the mutation on and off at a desired developmental stage or period of life. Conditional and inducible mutations are discussed at length in Chapter 12.

BREEDING STRATEGIES

To obtain large numbers of offspring for functional studies, heterozygote transgenic or knockout mice containing the desired gene mutation in the germ line are bred with normal mice. Several breeding strategies have been successfully employed. A variety of inbred and outbred strains of mice have been utilized for breeding. The goal of the breeding strategy is to optimize the expression of the mutation, maximize the number of viable offspring, and minimize the potential confounding influence of background genes from the breeder parents.

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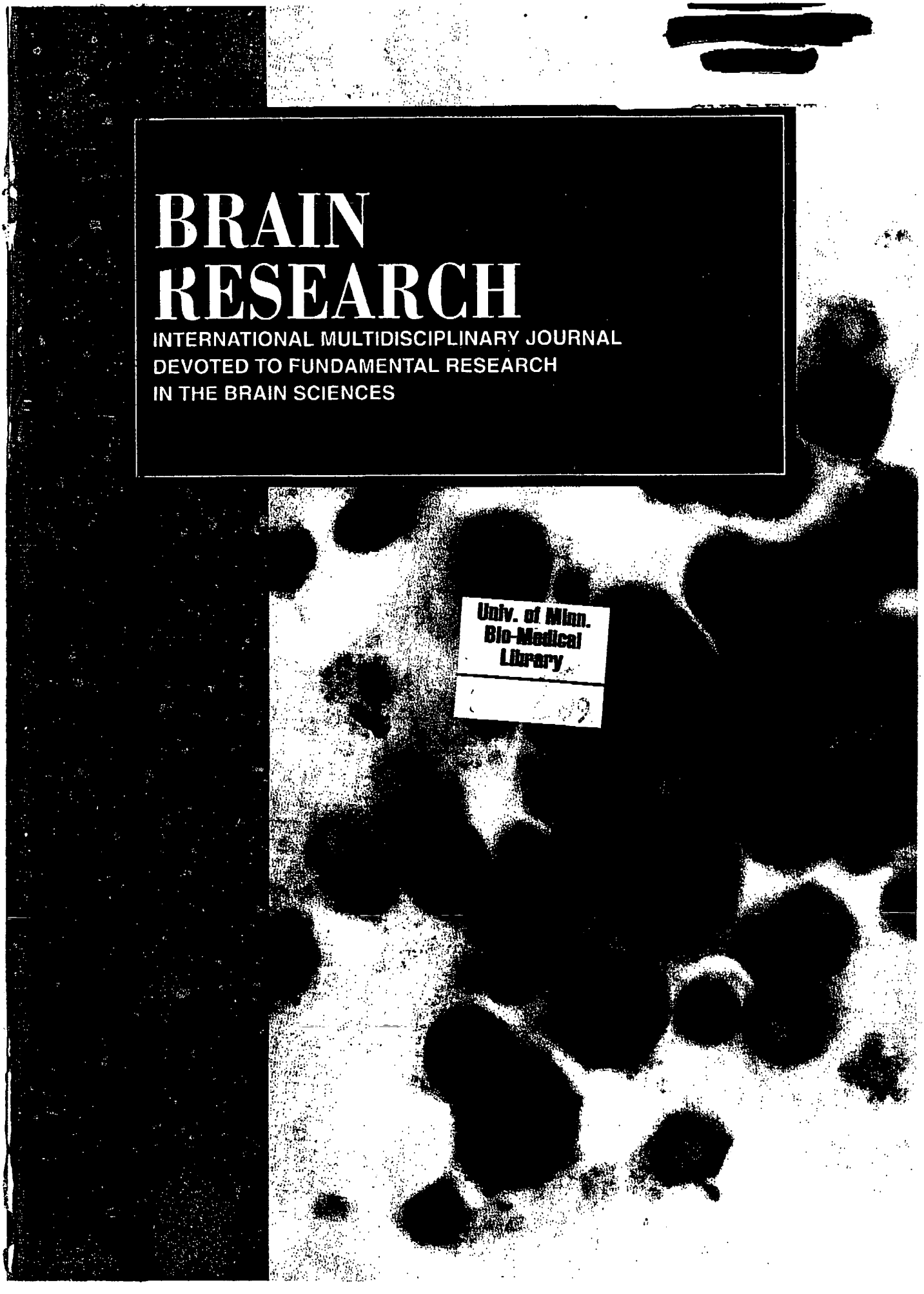

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Interactive report

Behavioral phenotyping of transgenic and knockout mice: experimental design and evaluation of general health, sensory functions, motor abilities, and specific behavioral tests [1]

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Abstract

Rigorous experimental design can minimize the high risk of false positives and false negatives in the behavioral phenotyping of a new transgenic or knockout mouse. Use of well established, quantitative, reproducible behavioral tasks, appropriate Ns, correct statistical methods, consideration of background genes contributed by the parental strains, and attention to litter and gender issues, will maximize meaningful comparisons of −/−, +/−, and +/+ genotypes. Strategies developed and used by our laboratory are described in this review. Preliminary observations evaluate general health and neurological reflexes. Sensory abilities and motor functions are extensively quantitated. Specific tests include observations of home cage behaviors, body weight, body temperature, appearance of the fur and whiskers, righting reflex, acoustic startle, eye blink, pupil constriction, vibrissae reflex, pinna reflex, Digiscan open field locomotion, rotarod motor coordination, hanging wire, footprint pathway, visual cliff, auditory threshold, pain threshold, and olfactory acuity. Hypothesis testing then focuses on at least three well-validated tasks within each relevant behavioral domain. Specific tests for mice are described herein for the domains of learning and memory, feeding, nociception, and behaviors relevant to discrete symptoms of human anxiety, depression, schizophrenia, and drug addiction. An example of our approach is illustrated in the behavioral phenotyping of C/EBPδ knockout mice, which appear to be normal on general health, neurological reflexes, sensory and motor tasks, and the Morris water task, but show remarkably enhanced performance on contextual fear conditioning.

1. Introduction

Targeted gene mutation technology represents a powerful new tool for biomedical research. When the targeted gene is expressed in the brain, the behavioral phenotype of the mutant mice may reveal genetic mechanisms underlying normal behaviors, and may increase our knowledge of genetic factors in neuropsychiatric disorders. *Transgenic mice* have a new gene, or an additional copy of an existing gene, added to the genome. *Knockout mice* have a targeted gene deletion, such that no product of the mutated gene is synthesized in the null mutants. The methods for developing the mutation, and breeding strategies to generate null mutants in the F2 and subsequent generations, are extensively described in this volume and elsewhere [11,54,76].

Approximately 100 different genes expressed in the central nervous system have been targeted and phenotyped in transgenic and knockout mice to date [4,11,52,54,68]. Reported behavioral phenotypes include aberrant social, reproductive, and parental behaviors, aggression, feeding disorders, learning and memory impairments, anxiety-like behaviors, and altered responses to antidepressants, antipsychotics, ethanol, and psychostimulant drugs of abuse. Experimental design is presently being optimized for thorough evaluation of behavioral phenotyping in mutant mice. This review is designed to suggest general methods that have been validated in our laboratory and others. Specific protocols for individual behavioral tasks can be found in the original publications referenced throughout the text, and in several recent reviews [11,16,19,20,34,38,54,74].

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2. The mice

After the mutation has been successfully introduced, and the gene product shown to be correctly overexpressed or absent, the first chimeras are mated with wildtype mice. A Mendelian distribution of F2 offspring is predicted. The strain of mouse used for the embryonic stem cells, for the donor blastulas, and for the breeding, can greatly affect the behavioral phenotype. For example, some 129 substrains (e.g. 129/J and 129/SvJ) have an incomplete or missing corpus callosum [42], and perform poorly on learning and memory tasks [16,76]. Background genes that are randomly contributed by each parental strain often interact with the mutated gene of interest, creating false positives [2,15,16,26,76]. For example, human Alzheimer's mutant β-amyloid precursor protein overexpressed in C57B6 breeder mice produces amyloid plaques in the brain and poor learning and memory on the Morris water task [31]. The same transgenic insertion in FVB/N mice did not show plaque formation and was lethal at too young an age for learning and memory testing. No single strain can solve all of the potential breeding problems. For some experiments, breeding into the 129/Sv strain is a good solution to unify the background genes by using the same strain for the embryonic stem cells and the parental breeders [39]. Other experiments benefit from a congenic breeding strategy into C57BL/6J, a standard, commercially available inbred strain that shows intermediate values on most behavioral phenotypes, has been characterized on a large number of behavioral tasks and is a reasonably prolific breeder [2,16,69].

Numbers of animals for standardized experimental designs and appropriate statistical tests are a minimum of $N = 10$ −/− null mutants, $N = 10$ +/− heterozygotes and $N = 10$ +/+ wildtype littermate controls. If a gender effect is detected, $N = 10$ of each gender and each genotype is required. Ages of the animals are approximately equivalent across genotypes in accordance with the goals of the experiment. For example, adult mice are best tested between ages 3 and 8 months; aged mice between 12 and 18 months; juvenile mice between 2 and 6 weeks.

3. Preliminary observations

A series of carefully conducted preliminary observations of general health, home cage behaviors, sensory abilities, and motor functions is first conducted for each mouse to avoid spurious false positives. If an animal has a major health problem or a gross motor defect it will be unable to perform many behavioral tasks for reasons not necessarily specific to the mutation. If an animal is blind or deaf, specific behavioral tests can be designed around the sensory deficit, such as olfactory learning tasks for blind mice or tactile startle tasks for deaf mice.

Our laboratory developed a set of preliminary observations and neurological reflexes which we use to assess gross defects in mutant mice [20]. This first step is likened to 'giving your mouse a physical exam.' The mouse is weighed, its body temperature is taken, and the appearance of its fur and whiskers is noted. Home cage locomotion, grooming, nesting, sleeping, and fighting patterns are recorded. Neurological reflexes are tested in each mouse. These include eye blink, ear twitch, whisker twitch, and righting reflex.

We next evaluate motor functions. Each mouse is tested for normal exploratory locomotion on the Digiscan open field [56]. The mouse is placed in the photocell-equipped automated open field box for a 5 min test session during which the Accuscan software calculates total distance traversed, number of movements, horizontal activity, vertical activity, and center/perimeter time. Each mouse is next tested for motor coordination on the Basile automated accelerating rotarod [33,67]. The mouse is placed on the cylinder and the speed of the cylinder rotation is gradually accelerated from 4 to 40 revolutions per min over a 5-min period. Latency to fall from the rotarod is recorded. The fall is approximately 6 inches, a height that mice can easily fall and land on their feet without injury. Neuromuscular strength is tested by the wire hang test [60]. The mouse is placed on a wire cage lid and the lid is gently waved in the air so the mouse grips the wire. The lid is then turned upside down, approximately 6 inches above the surface of soft bedding material. Latency to fall onto the bedding is recorded, with a 60 s cut-off time. Gait is measured with the footprint test [3]. The two hindpaws are dipped into black ink. The mouse is then immediately placed onto white paper in a dark narrow tunnel approximately 12 inches long. The ink is then wiped off the feet and the pattern and pathway of the black footprints on the white paper are calculated.

Sensory functions are then assessed. Visual ability is measured in the visual cliff test conducted in a box with a ledge. The inner surface of the box and ledge are covered with black and white checkerboard contact paper which emphasizes the ledge drop-off. A piece of clear plexiglas spans the ledge so that there is no actual drop-off, just the visual appearance of a cliff. Normal mice will stop at the 'edge' and explore the plexiglas floor before walking forward. Blind mice will not see the appearance of the edge and will walk forward across the plexiglas immediately. Hearing is assessed with the acoustic startle test [59]. The reflexive flinch and eyeblink to a sudden loud noise comprise the standard acoustic startle response. An automated startle system is used to deliver the startle stimulus and measure the response of the mouse. The mouse is placed in a small cylindrical restraining tube within a sound-attenuating chamber. Stimulus tones are varied randomly from 70 dB to 120 dB, 40 msec duration, in the presence of background white noise at 70 dB. A standard battery of tones begins at threshold sound levels of 72 and 75 dB, includes moderately loud tones of 80, 90, and 100 dB, and ends at the loudest sound levels, 110, 115, and

120 dB. Whole body flinch amplitude is automatically recorded by the pressure transducer. Tactile startle is measured by whole body flinch amplitude to a brief puff of air delivered by the system to an area near the face. A typical session lasts for about 30 min, after which the mouse is returned to the home cage. Sense of touch can be measured with Von Frey hairs, made of thin wires which are used to gently touch the footpads, to measure the reflexive twitch to touch. A simple olfactory test can be used, e.g. latency to locate an odiferous piece of food, e.g. a piece of cheese or smear of peanut butter, buried under the litter in a clean test cage, or time spent sniffing a novel odor such as vanilla extract painted onto the test cage wall.

4. Specific behavioral tasks to test discrete hypotheses

Targeted gene mutation is designed to address specific hypotheses about the behavioral role of a gene. The set of specific behavioral tasks to be used for behavioral phenotyping is designed around the hypothesis. Genes for neuropeptides in the hypothalamus might be analyzed in tests of feeding behaviors, sexual behaviors, and stress-related behaviors. Signalling genes expressed in the hippocampus might be analyzed in tests of spatial learning and memory. Genes for serotonin receptor subtypes might be analyzed in tests of aggression, feeding, and mouse behaviors relevant to symptoms of human depression. A thorough knowledge of the behavioral literature is required to choose the optimal constellation of behavioral tests to address the critical hypotheses.

In some cases, tests designed for rats can be directly used for mice. For example, the Digiscan automated open field works equally well for rats and mice, and the resident/intruder procedure for aggressive behaviors is applicable to males of both species. In some cases, the equipment can be modified for mice often simply by building a smaller version of the rat test apparatus. For example, a mouse rotarod and a mouse elevated plus maze are downsized versions of the rat rotarod and rat elevated plus maze. In some cases, the equipment must be changed to accommodate the behavior of the mouse. For example, lighter weight levers allow a mouse to conduct a lever press task in an operant chamber; alternatively, a nose-poke insert replaces the lever in an operant chamber to accceleraate the autoshaping process, because mice tend to explore dark holes more readily than they press levers. In some cases, mouse and rat behaviors are too dissimilar. For example, social interaction is easily measured between two rats placed together in a new environment, but mice will primarily explore the new environment rather than interacting each other. Rats can perform complex operant tasks which appear to be far beyond the abilities of mice.

This article provides an overview of several behavioral tasks that our laboratory and others have validated for mice. The tasks described in this review represent only a small subset of the applicable behavioral tests available in the wider behavioral neuroscience literature. The reader is referred to the many excellent chapters in this volume which describe multiple tasks in specific behavioral domains in much greater detail.

Because each task has its own limitations, it is best to employ multiple tasks for each behavioral domain of interest to avoid false negatives. For example, a mutation in a gene expressed mainly in the amygdala may produce performance deficits on fear-conditioned learning tasks, which require an intact amygdala, but not on spatial navigation learning tasks, which require an intact hippocampus. When sensory or motor deficits are detected during the preliminary screen, the choice of the multiple tasks is limited by the physical disability of the mutants. Knowledgable behavioral neuroscientists can generally identify two or three good tasks for each type of behavior relevant to each hypothesized function of the gene product.

4.1. Feeding

Twenty-four hour consumption of normal rat chow in the home cage is measured by daily weighing of the food [23,32]. Standard amounts of food are provided by the investigator. Another approach is a challenge test, measuring consumption of a palatable food source or specific macronutrients during a test session [17]. A high carbohydrate/high fat source, such as sweetened condensed milk, cookie mash, or a commercially available powdered chow diet, is provided in a clean test cage. The mouse is placed in the cage for the fixed time period. Alternatively, a two-choice paradigm involves presentation of the special diet in a second food hopper next to the standard chow in an identical food hopper, or presentation of test liquid solutions in separate water bottles, attached to the home cage [5,7]. Food or water restriction may be performed in special circumstances, e.g. to study a gene that regulates consumption only after an overnight fast, or as a necessary component of food or water reinforcement in learning and memory tasks.

4.2. Learning and memory tasks

Morris swim tasks [51] have been standardized to measure spatial navigation learning and memory in mice [16,71,76]. Each mouse learns to swim in a circular pool of water to locate a submerged hidden platform. The water level is 30 cm deep, maintained at 24–26°C with aquarium heaters, and changed daily. Non-toxic Crayola white paint is added to make the water opaque and the platform invisible to the mouse. An automated video tracking software system is used to quantitate swim speed, swim pathway, latency to reach the platform, and time spent in each of the four quadrants of the pool. Mice are excellent swimmers and readily dry themselves off and groom when removed from the pool. While additional drying or heat is

usually not necessary, a soft cloth is available to dry the mice and a hot plate is available to place under the cage, if needed. During training on the visible platform task, the platform is raised above the surface of the water, or a prominent cue is attached to the platform to extend above the surface of the water. The mouse is gently guided to the platform by the experimenter and allowed to remain on the platform for 20 s. The mouse is then placed along the edge of the pool, facing the wall, in one of four randomized start locations equidistant around the perimeter. The mouse is given 60 s to swim to the visible platform and allowed to remain on the platform for 20 s. Latency to find the visible platform over four successive trials is recorded. Mice that do not swim to the visible platform are noted, and given further guided training by the experimenter. Any mouse that does not swim, and any mouse that only floats, is eliminated from the experiment. Mice that learn to navigate to the visible platform are then trained on the hidden platform task. The platform is submerged below the surface of the water, so that the mouse cannot see the location of the platform. Each trial begins with the mouse being placed in the pool, facing the wall, in a different quadrant on each trial. Most acquisition training on the hidden platform task consists of two or three blocks a day of four trials per block = 8 to 12 trials per day, for three to five consecutive days. Fewer trials per day may be used for mice that learn rapidly. More days may be needed for mice that learn slowly. After the acquisition curve reaches the performance criteria (usually 10–20 s latency to find the hidden platform), the probe trial is administered. The platform is removed from the pool. The mouse is placed in the pool for a single 60 s trial. Time spent in the trained quadrant, where the platform was previously located, is compared to time spent in the other three quadrants, to confirm acquisition. If time spent in the trained quadrant is significantly greater than time spent in each of the other quadrants, the mouse has learned the location of the hidden platform, rather then having learned an effective general search strategy. The probe trial is generally administered at the end of the last training day. Additional probe trials may be administered on subsequent days, as a measure of retention. Additional training may precede subsequent probe trials. Another approach is to test reversal learning, in which the mouse is retrained with the hidden platform in another quadrant. Probe trial testing is similarly conducted after reversal training.

Cued and contextual conditioning is a fear-conditioning task that measures memory of an aversive experience, and the stimuli present during the aversive experience [10,16,24,37,71,75]. A standard footshock shuttle box, or a specialized automated freeze monitor and software package, is used to control footshock delivery and to measure duration of freezing behavior. Freezing, a standard response to a sudden aversive stimulus, is defined as complete behavioral immobility except for respiration. On day 1, the mouse is placed in a chamber and allowed to explore for 2 min. The chamber is square and illuminated by normal house lighting. An auditory stimulus (80 dB white noise) is then presented for 30 s. One footshock, 0.5 mA, 2 s duration, is then delivered. The animal is then left in the cage for 30 s, then returned to its home cage. On day 2, the mouse is returned to the same testing chamber. The time spent in freezing behavior is measured over a 5-min period. Total number of seconds spent in complete behavioral immobility are tallied. One hour later the mouse is placed into the same testing chamber, but the context has been changed. The grid floor is now covered with plexiglas, the square is divided by a piece of plexiglas to form a triangle-shaped chamber, a red chamber light is on, and an odor is painted onto the chamber walls, e.g. vanilla or lemon extract. Freezing is quantitated for 3 min. Then the auditory cue is presented. Freezing is quantitated over the next 3 min, in the presence of the 80 dB white noise cue. The mouse is then returned to its home cage. Thus, memory is assessed by measuring freezing under three conditions for each mouse: context, altered context, and auditory cue. Chambers are thoroughly cleaned after each test session.

Maze learning and avoidance tasks are among the oldest learning and memory tasks used in rodents. Passive and active avoidance tasks measure memory of an aversive experience, through simple avoidance of a location in which the aversive experience occurred [8,44,57,67]. A commercially available automated apparatus consists of two connected chambers, one lighted, one dark. Mice tend to prefer a dark environment and will immediately enter the darkened chamber. Passive avoidance is a two day task. On day 1, the training session, the mouse is placed in the lighted chamber for 10 s. The door to the dark chamber is then opened, and latency to enter the dark chamber is measured as a control for visual ability and motor activity. Immediately after the mouse enters the dark chamber, a 0.3 mA, 1 s footshock is delivered. The mouse remains in the dark compartment for 10 s after the shock to allow formation of the association between the dark compartment and the footshock. The mouse is then returned to the home cage. On day 2, the retention test session, the mouse is placed in the lighted compartment and the door is opened. Latency to enter the dark compartment is measured, with a 300 s cut-off time. The mouse is then returned to its home cage. An additional exposure to the single footshock on day 2, followed by latency to enter the dark on day 3, can be used to obtain additional information on acquisition of passive avoidance in mice with apparent poor acquisition. For active avoidance, using the same chamber, with the same parameters, the mouse must move into the opposite chamber to avoid receiving a footshock. Latency to enter the non-shocked chamber is the measure of learning.

Spatial maze tasks include the T-maze, Y-maze, radial mazes, and Barnes maze [21,30,58]. Acquisition of location of a reinforcer over repeated trials provides the mea-

sure of learning and memory. The Barnes maze is a circular platform, 1.3 meters in diameter, in which 36 holes, each 8 cm in diameter, are equally spaced around the perimeter of the circle, 10 cm from the edge. Mice tend to poke their noses into holes, especially to explore dark holes which may lead to escape routes away from a lighted, open field. One of the holes leads to a dark, enclosed box, located just below the circular platform. The mouse is placed in the center of the platform under a start box. The start box is lifted and the mouse is guided to the escape tunnel where it remains for 1 min. Then the mouse is placed back in the start box and allowed to explore the maze. When the mouse finds the correct hole it escapes into the dark box. The mouse is allowed to remain in the escape box for 1 min then removed from the box and given repeated trials in which it is placed in the center of the platform and allowed to explore for 5 min per trial, 1–4 trials per day, for up to 20 training days. Time to locate the tunnel hole, distance travelled, and number of errors on each training day (noses pokes into the incorrect hole) provide the measures of acquisition.

The T-maze is shaped like the letter T, with either the left or right arm containing concealed cups. One Noyes food pellet or 0.05 ml tap water is used for food or water reinforcement, respectively, in food or water restricted mice, respectively. In the continuous reinforcement procedure, the mouse is placed in the start end of the T, allowed to explore the maze and obtain the reinforcer. The mouse must then return to the start box and choose the opposite arm on the next trial to obtain the reinforcer. Twelve trials per day are conducted for up to 10 consecutive days. Acquisition of the procedure and choice accuracy in this alternation task are the measures of procedural and learning abilities.

Operant tasks are more difficult for mice than for rats, but mice can learn some lever press acquisition tasks [48,64]. Food or water restricted mice are habituated to the operant chamber and shaped to press the lever for the food (small food pellet) or water (0.05 ml tap water) reinforcer, respectively. Number of trials to acquire the lever press task is the simplest measure of learning in mice. The more complex non-matching to position task requires the mouse to receive reinforcement for pressing the opposite lever to the lever illuminated on the previous trial by the cue lamp above the lever. A discriminative go–no go task for mice requires the mouse to press the lever only when the cue light is illuminated to obtain the reinforcement, or to press the lever only during the period in which a tone is sounding. Modifications of the rat operant chamber for mice include levers that are smaller and more sensitive to the touch of the mouse paw, and a module which substitutes the lever for a nose-poke into a dark hole, to obtain the reinforcement

Motor learning is evaluated by repeated daily testing on the rotarod [60]. Reduction in latency to fall, over repeated testing sessions, provides a measure of cerebellar learning.

4.3. Pain sensitivity tests

The two methods to assess analgesia that have been well validated and standardized for mice are the tail flick and hot plate tasks [22,62,66]. Tail flick nociception measures a simple spinal reflex to a sudden, painful thermal stimulus. A photobeam is used to apply a heat stimulus to the tail. Latency to flick the tail out of the path of the light beam is measured. The photobeam is turned off if the tail is not flicked away within 15 s, to avoid tissue damage.

Hot plate nociception is a similar reflex which requires higher brain centers. The mouse is placed on the surface of a hotplate which is maintained at 50–55°C. A plastic frame encloses the surface so that the mouse cannot jump out. Latency for the mouse to raise and lick its forepaw, or to jump up, is recorded. The mouse is then immediately removed from the hotplate. If the mouse has not responded within 30 s, it is removed from the hotplate, to prevent tissue damage.

Pain sensitivity to footshock is assessed by threshold analysis as a control for procedures requiring a footshock, e.g. some of the learning and memory tasks described above. A sequence of single footshocks is delivered in the avoidance chamber and the mouse is observed for flinching, jumping, running, and vocalization. When these behaviors are observed the sequence is terminated. The sequence begins with 0.075 mA and proceeds through 0.1 mA, 0.15 mA, 0.25 mA, and 0.35 mA, and 0.45 mA single footshocks of 1 s duration each. Most mice show behavioral responses to the lower footshock levels and therefore do not receive the higher footshocks.

4.4. Anxiety-related behaviors

Light ↔ dark exploration is an ethologically-based approach-avoidance conflict test which is sensitive to anxiolytic drug treatments [14,16,18,45]. Mice prefer a dark, enclosed, small space over a brightly lit, open, large space. However, mice are also highly exploratory. The light ↔ dark exploration task represents a naturalistic conflict between the tendency of mice to explore a novel environment versus the tendency of mice to avoid a brightly lit open field. The mouse is placed into the large, lighted compartment of a two-compartment chamber. The mouse repeatedly enters the adjoining small, dark, enclosed chamber, then emerges back into the larger, lit, open chamber. Number of transitions between the two chambers and time spent in each chamber are automatically recorded through a photocell array across the border between the two chambers over a single 10-min test session.

The elevated plus maze task similarly measures the conflict between exploration of a novel environment and avoidance of brightly lit open areas [25,41,72,70]. An additional factor is that the surface is raised 1 meter from the floor. The elevated plus maze is in the shape of a +. Two alternate arms are dark and enclosed, while two

alternate arms are open, lit, and without edges. The mouse is placed in the center of the + and allowed to explore the maze for 5 min. Numbers of entries into the open arms versus number of total arm entries, and time spent on the open arms versus the closed arms, provide the measures of anxiety-related behavior.

The Digiscan open field measure of center time versus perimeter time gives a less specific measure of anxiety-related behaviors. In a brightly lit open area, mice will tend to stay near the walls of the open field rather than enter the center region. Habituation to the novelty of the open field, i.e. reduction of perimeter time and increase in the center time, is measured by recording activity in 5 min consecutive intervals over a 1-h period. Repeated 1 h tests over several days gives a further measure of habituation to the aversive properties of the novel open field. While the open field test is not as specific for anxiety-related behaviors, the center time parameter provides some indications that can be used as a starting point for further testing on the more specific anxiety-related tests.

4.5. Depression-related behavior

The Porsolt swim test is used to evaluate "behavioral despair," a measure of failure to seek escape from an aversive stimulus [63]. The mouse is placed in a cylinder of room temperature tap water and swimming behavior is observed over a 10-min test session. The water is approximately 12 inches deep, such that the animal cannot balance on its feet or tail. The water surface is approximately 6 inches from the top of the cylinder, such that the animal cannot jump out. Rodents will generally swim. Animals treated with certain drugs or lesions will stop swimming and will float. Floating time is considered the measure of depression-like behaviors, in that the animal has stopped swimming and 'given up' on finding an escape route. Floating time is decreased by treatment with antidepressant drugs. Any mouse that does not swim or float is immediately removed from the water. At the end of the 10 min swim test the mouse is dried with a towel and returned to its home cage.

4.6. Schizophrenia-related behavior

Deficits in prepulse inhibition are common in schizophrenic patients and may measure attentional dysfunctions that contribute to auditory hallucinations [27]. Prepulse inhibition is a sensorimotor gating reflex, similarly quantitated in mice, rats, and humans [27,59]. When the startle stimulus is immediately preceded by a milder stimulus, delivered immediately before the startle stimulus, the mouse will flinch less to the startle stimulus. Prepulse tones of 74, 82, or 90 dB are randomly presented 100 msec before each 100 dB or 120 dB startle tone. Whole body flinch amplitude is again automatically recorded. To evaluate a separate sensory modality, a puff of air is used as the prepulse, before the acoustic or tactile startle stimulus.

4.7. Social behaviors

A variety of social behaviors can be quantitated by videotaping the mice over 24 h periods in the home cage [39]. Observations during the night are conducted under red light, which does not disturb the mice. Group huddling while sleeping is one characteristic of normal mouse social behavior. Nest building can be observed over a 1-h period after placing a Nestlet cotton square or other nesting material in the floor of the home cage. Depth of nest provides a quantitative measure of nest building. Fighting can be quantitated from the videotapes by scoring number and duration of attacks.

The tube test for social dominance can be used to evaluate aggressive behaviors [39,40]. Two mice are placed in opposite ends of a plexiglas tube, 3 cm in diameter, 35 cm long. The mouse that advances beyond the midpoint is considered the dominant male. The mouse that backs away from the midpoint is considered the subordinate male. Mice are removed immediately after scoring. Fighting does not occur.

Isolation-induced standard opponent and resident-intruder testing for aggression in male mice is conducted according to established methods [16,49,53]. A standard subordinate test opponent male is placed in the cage of the test male. The test male has been singly housed for approximately one month. Attacks over a 5 min test session by the test male are scored for latency to first attack, number of attacks, number of bites, chasing, and tail-rattling.

4.8. Sexual and reproductive behaviors

Mouse sexual and parental behaviors can be scored using standardized methods [12,55,65,77]. Male sexual behavior is quantitated by observational methods for latencies and frequencies of mounts, intromissions, and ejaculations. Female sexual behavior is quantitated by standardized observations methods for the lordosis response. Pregnancies and number of pups delivered per pregnancy are measures of reproductive success. Lactation can be scored by an observer. Presence of milk in the stomach of the pup can be ascertained in cases of early postnatal death. Parental behaviors are measured by quantitating retrieval of pups to the nest and sitting with the pups in the nest. Before weaning, isolated mouse pups emit ultrasonic vocalizations which alert the parent that the pup is out of the nest and triggers retrieval behavior. Olfactory cues are used by the parents to identify their own pups. Quantitating parental behaviors provides a good example of the need to test sensory abilities before conducting a specific test. If sensory abilities are not tested, the parent may be deaf or

anosmic due to the mutation, and deficits in parental behaviors can be overinterpreted [9].

4.9. Addictive drugs

Good paradigms are available to assess responsivity to drugs of abuse in mice. Self-administration of drugs of abuse, including alcohol, cocaine, morphine, and nicotine, are measured in mice [1,5,7,13,28,29,43,46,50,61]. Drugs are self-administered intravenously or in the drinking water. Conditioned place preference quantitates the rewarding internal cues created by a drug treatment. Measures of tolerance, dependence, and withdrawal symptoms are quantitated by an observer, using standardized scoring methods.

5. Conclusions

In the opinion of this author, the present technology for the generation of mutant mice is most useful for modeling the symptoms of single gene mutation human hereditary diseases. Behavioral phenotypes identified for transgenic and knockout mice may lead to important medical applications. When the mutation is designed to mimic the genetic mutation in a human hereditary disease, the mouse behavioral phenotype can serve to evaluate the efficacy of new pharmacological and gene therapy treatments [4,6,11,56]. Behavioral phenotypes revealed in the mutant mouse model can thus serve as quantitative surrogate markers to test the efficacy of potential therapeutics.

Two major problems of the current transgenic and knockout technology limit its usefulness to the more general study of the role of genes in normal and abnormal behaviors. The first is the presence of the mutation in all cells. A gene expressed in the brain may also be expressed in many peripheral organs. It is impossible to assign a behavioral difference in the mutant mouse to a specific brain structure or pathway, or even to the nervous system. Tissue-specific 'conditional' knockouts are being generated to solve this problem ([35,36,47,73], and see other reviews in this volume). The second is the presence of the mutation from the earliest stages of development. Other genes may compensate during development for the absence of the deleted gene or for the overexpression of the transgene. Lack of behavioral phenotype may be due to compensation by a redundant gene which takes over the function of the missing gene. An opposite behavioral phenotype to that predicted may be due to overcompensation by one or more redundant genes. An observed behavioral phenotype may in fact characterize the role of the compensatory gene, not the targeted mutation. Temporally-selective 'inducible' transgenics are being generated to solve this problem ([36,47], and see other reviews in this volume).

If conditional and inducible mutations become feasible, it is likely that the transgenic and knockout technology will become the premiere research tool to further our understanding of the genetic basis of behavior. Gene mutations may replace pharmacological tools, such as drugs that act as selective antagonists at neurotransmitter receptor subtypes. Gene mutations may also replace lesions of brain structures or pathways to study the functions of endogenous neurochemicals and neuroanatomical pathways. Behavioral phenotyping methods continue to evolve, to optimize our approaches to the growing opportunities of the mutant mouse technology.

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